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Over-estimation of Glucose-6-phosphatase Activity in Brain *in Vivo*

APPARENT DIFFERENCE IN RATES OF [2-^{3}H]GLUCOSE AND [U-^{14}C]GLUCOSE UTILIZATION IS DUE TO CONTAMINATION OF PRECURSOR POOL WITH ^{14}C-LABELED PRODUCTS AND INCOMPLETE RECOVERY OF ^{14}C-LABELED METABOLITES*

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Significant dephosphorylation of glucose 6-phosphate due to glucose-6-phosphatase activity in rat brain *in vivo* was recently reported (Huang, M., and Veech, R. L. (1982) *J. Biol. Chem.* 257, 11358–11363). The evidence was an apparent more rapid ^{3}H than ^{14}C loss from the glucose pool and faster [2-^{3}H] glucose than [U-^{14}C]glucose utilization following pulse labeling of the brain with [2-^{3}H,U-^{14}C]glucose. Radiochemical purity of the glucose and quantitative recovery of the labeled products of glucose metabolism isolated from the brain were obviously essential requirements of their study, but no evidence for purity and recovery was provided. When we repeated these experiments with the described isolation procedures, we replicated the results, but found that: 1) the precursor glucose pool contained detritiated, ^{14}C-labeled contaminants arising from glucose metabolism, particularly 2-pyrrolidone-5-carboxylic acid derived from [^{14}C] glutamine; 2) [^{14}C]glucose metabolites were not quantitatively recovered; 3) the procedure used to isolate the glucose itself produced detritiated, ^{14}C-labeled derivatives of [2-^{3}H,U-^{14}C]glucose. These deficiencies in the isolation procedures could fully account for the observations that were interpreted as evidence of significant glucose 6-phosphate dephosphorylation by glucose-6-phosphatase activity. When glucose was isolated by more rigorous procedures and its purity verified in the present studies, no evidence for such activity in rat brain was found.

It was recently reported by Huang and Veech (1, 43) that glucose-6-P[1] is dephosphorylated by glucose-6-Pase (EC 3.1.3.9) activity in rat brain *in vivo* at a rate equal to 35% of its rate of formation. This surprising finding has provoked considerable attention because brain is generally considered not to be a gluconeogenic organ, and brain glucose-6-Pase activity assayed *in vitro* is only a small fraction of that found in known gluconeogenic organs, such as kidney and liver (2–5). The procedure used by them to determine glucose-6-P dephosphorylation in brain *in vivo* (1) was to pulse label the brains of rats with [2-^{3}H,U-^{14}C]glucose by intracarotid injection, remove the brains by freeze-blowing at various times after the pulse (6), determine the specific activities of the ^{3}H- and ^{14}C-labeled glucose isolated from the brain tissues and plasma, and measure the amounts of ^{3}H- and ^{14}C-labeled metabolites formed in the brain in the interval between the pulse and the killing. From these measurements the ^{3}H/^{14}C ratios of the glucose isolated from brain and plasma and the rates of utilization of [2-^{3}H]glucose and of [U-^{14}C]glucose in brain were calculated. The principle of this method is based on the selective loss of the ^{3}H during the conversion of glucose-6-P to fructose-6-P by glucose-6-P isomerase (EC 5.3.1.9) (7). Dephosphorylation of the detritiated but still ^{14}C-labeled glucose-6-P derived from the rapid reversibility of the isomerase step would then return [^{14}C]glucose without the ^{3}H label back to the glucose pool. This transfer of ^{14}C from product back to precursor would diminish the net production of ^{14}C-labeled metabolites by an equivalent amount and keep the specific radioactivity of the precursor [^{14}C]glucose pool from falling as rapidly as it otherwise would. Because ^{3}H is irreversibly lost at the isomerase step to ^3H_2O, the principal ^{3}H-labeled metabolite of [2-^{3}H]glucose, such dephosphorylation would affect neither the production of ^{3}H-labeled metabolites nor the specific activity of the [^{3}H]glucose pool. The rate of glucose utilization calculated from ^{3}H-labeled metabolite formation and [^{3}H]glucose specific activity would then be greater than that calculated from ^{14}C-labeled metabolite formation and [^{14}C]glucose specific activity. Also the ^{3}H/^{14}C ratio of the glucose isolated from brain would fall with time. These are the effects reported by Huang and Veech (1).

The validity of this experimental design rests on the purity of the glucose fractions isolated from brain in which the specific activities of [^{3}H,^{14}C]glucose and ^{3}H/^{14}C ratios are determined and on complete recovery of the labeled metabolites of [2-^{3}H,U-^{14}C]glucose. At first, Huang and Veech (1) assayed the glucose specific activities in eluates from Dowex 1 borate columns (obtained after passage of brain extracts through tandem Dowex 1 formate/Dowex 1 borate columns). In a subsequent study (43), they derivatized the glucose in the eluates with hexokinase, separated the glucose-6-P derivative on Dowex 1 formate, and assayed the specific activities in fractions eluted from the Dowex 1 format columns. Some

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[1] The abbreviations used are: glucose-6-P, glucose 6-phosphate; fructose-6-P, fructose 6-phosphate; deoxyglucose-6-P, 2-deoxy-D-glucose 6-phosphate; glucose-6-Pase, glucose-6-phosphatase; hexose-P, hexose phosphate.

carbohydrate products of glucose metabolism beyond fructose-6-P (*e.g.* glycogen, glucosamine, and fructose) bind to Dowex 1 borate columns (8–10), and all would have lost ^3H but not ^{14}C. Some of them can be phosphorylated by hexokinase (11, 12) and would then co-purify with the glucose-6-P. It was, therefore, not surprising that these results were not confirmed in similar experiments in which different procedures for the isolation and purification of the labeled glucose were used (13).

Because contradictory results obtained with different methods are often construed as controversial and leave issues unresolved, we have repeated the experiments of Huang and Veech (1, 43), but examined each step in their procedures in detail. Our results show that glucose isolated from brain by these procedures is contaminated with detritiated, ^{14}C-labeled products derived from [2-^3H,U-^{14}C]glucose metabolism, and ^{14}C-labeled metabolites are not fully recovered in the product fractions. These findings explain the observations of Huang and Veech (1, 43) without invoking glucose-6-pase activity. When purification was carried out and verified with alternate procedures, no evidence of glucose-6-P dephosphorylation in rat brain was detected.

EXPERIMENTAL PROCEDURES

Materials—D-[2-^3H]Glucose (24 Ci/mmol), D-[U-^{14}C]glucose (258 mCi/mmol), 2-[1-^{14}C]deoxy-D-glucose (44 mCi/mmol), L-[U-^{14}C]glutamine (280 mCi/mmol), L-[U-^{14}C]alanine (176 mCi/mmol), and D-[1-^{14}C]glucosamine-HCl (54.2 mCi/mmol) were purchased from Du Pont-New England Nuclear. L-[4,5-^3H]Leucine (147 Ci/mmol) was obtained from the Amersham Corp. Hexokinase (EC 2.7.1.1, yeast), glucose-6-P dehydrogenase (EC 1.1.1.49, yeast), glucose oxidase (EC 1.1.3.4, *Aspergillus niger*), and catalase (EC 1.11.1.6, beef liver) were obtained from Boehringer Mannheim. Ion exchange resins, Dowex AG 50W-X8-H^+ (200–400 mesh) and Dowex AG 1-X8 formate (200–400 mesh) were purchased from Bio-Rad. X-ray film (SB-5) for autoradiography and thin layer flexible chromatographic plates (13255 cellulose) were obtained from Kodak.

Dowex 1 borate columns were prepared by passing 25 ml of fresh 0.12 M sodium tetraborate (Sigma) through 1 × 0.8-cm Dowex AG 1-X8 formate columns at room temperature and washing the columns twice with 25 ml of water first at room temperature and then again at 4 °C.

Animals—Sprague-Dawley male rats (210–280 g; Taconic Farms, Germantown, NY) were used. Preparation of the animals and the experimental procedure were the same as those of Huang and Veech (1). Under pentobarbital anesthesia a polyethylene catheter (PE-10, Clay-Adams, Parsippany, NJ) was inserted into the left external carotid artery, advanced into the common carotid artery until the tip was 5 mm caudal to the carotid bifurcation, and secured with ligatures; the free ends of the catheters were passed under the skin and exteriorized at the nape of the neck. The right internal carotid artery was ligated. The animals were then allowed to recover and provided food and water *ad libitum* for approximately 24 h. The experimental procedure was begun by injection of a 50-μl pulse of 5.1 mM glucose, 0.9% (w/v) NaCl, containing approximately 50 μCi of D-[2-^3H]glucose and 5 μCi of D-[U-^{14}C]glucose, into the catheterized carotid artery; the $^3H/^{14}C$ ratio of the injectant was measured and found to be 9.4. The rats were unrestrained during the injection and most of the procedure but were transferred to plastic restrainers and positioned in the apparatus at about 1 min before being killed by freeze-blowing of the brains (6) at various times between 3 and 9 min after the pulse. Immediately after the killing, blood samples were drawn by cardiac puncture and centrifuged to obtain the plasma. Brains and plasma were stored at −80 °C until processed further.

Processing of Brain Tissue and Plasma—Frozen brains were powdered under liquid nitrogen in a cryostat maintained at −30 °C and divided into weighed 100–200-mg portions. Brain and plasma (100 μl) samples were thawed in 3 volumes of 3 M perchloric acid at −12 °C, diluted with water to 0.6 M $HClO_4$, centrifuged to remove precipitated protein, neutralized to pH 7.0–7.5 with 2.0 M KOH, 0.4 M potassium acetate, 0.4 M imidazole (14), and centrifuged to remove the $KClO_4$ precipitate. Samples of the injection solution were mixed with non-radioactive neutralized acid extracts of brains from uninjected rats and processed further in parallel with the extracts from the injected rats. Glucose was isolated from portions of these neutralized extracts.

Isolation of Labeled Glucose by Tandem Dowex 1 Formate/Dowex 1 Borate Column Chromatography—The procedure used by Huang and Veech (1) to isolate glucose from neutralized acid extracts of brain tissue was followed as closely as possible (Procedure A). Although not specified in their report, in the present studies all chromatographic columns and solutions applied to them were kept at 4 °C because alkaline rearrangements of sugars can occur in sugar-borate complexes at room temperature (15, 16) and result in detritiation of [2-^3H]glucose. Portions (0.5 ml) of the neutralized acid extracts of brain and plasma were applied to tandem Dowex 1 formate columns (0.8 × 5 cm)/Dowex 1 borate columns (0.8 × 1 cm). The columns were then washed with 15 ml of water and the combined effluents and washes collected. The tandem columns were then separated and individually eluted with 10 ml of 4 N formic acid (1). The Dowex 1 borate column eluates were collected in vials and dried in a stream of air at 70–75 °C (1); the boric acid was removed as methyl borate by dissolving the dried eluates in 5 ml of absolute methanol and air drying again at 70–75 °C (1); the residues were redissolved in 1 ml of water. Portions of the redissolved Dowex 1 borate column eluates were examined for purity by TLC and amino acid analysis and then further purified as described below. Portions of the tandem column effluents, Dowex 1 formate column eluates and redissolved Dowex 1 borate column eluates were assayed for ^3H and ^{14}C content by liquid scintillation counting (Beckman Model LS-5801, Fullerton, CA) with external standardization of counting efficiency. Sufficient counts were collected to achieve a coefficient of variation of the counting rates for both ^3H and ^{14}C of no greater than 2%.

Subsequently, Huang and Veech (43) modified their procedure for purifying glucose from the tissue extracts; they derivatized the glucose in the Dowex 1 borate column eluates with hexokinase, and the glucose-6-P was separated on Dowex 1 formate columns. We therefore modified Procedure A by adding the following derivatization and separation steps (Procedure B). Portions (0.5 ml) of the dried, redissolved eluates from the Dowex 1 borate columns were incubated with 0.28 units of yeast hexokinase in a final volume of 1 ml containing 0.8 mM $MgCl_2$, 0.25 mM ATP, and 80 mM Tris buffer, pH 8.1, for 45 min at room temperature (14).[2] The reaction mixtures were cooled to 4 °C and applied to Dowex 1 formate columns (0.8 × 5 cm). The columns were washed with 15 ml of water and then eluted with 10 ml of 8 N formic acid. The two eluates were collected separately, dried under a stream of air at 70–75 °C, and redissolved in water. Portions of each of the eluates were assayed for ^3H and ^{14}C as above and examined for purity by TLC and amino acid analysis.

Thin Layer Chromatography—One-dimensional and/or two-dimensional TLC was used to examine the radiochemical purity of eluates from the Dowex columns. Eluates from the Dowex 1 borate columns, after removal of the borate as methyl borate, were chromatographed on flexible cellulose plates (Kodak 13255 cellulose) in the ascending direction with 23 M formic acid:*t*-butanol:methylethyl ketone:water (15:40:30:15, v/v/v/v) as the solvent (Solvent I) (17). Glucose and selected other carbohydrate and amino acid standards, as well as the [2-^3H,^{14}C]glucose injection solution, were chromatographed in parallel lanes. For quantitative analysis the chromatograms were dried, cut into strips (1 cm × 0.5 or 1 cm), placed in counting vials containing 1 ml of water, vigorously mixed, and assayed for ^3H and ^{14}C as above. Sugars were visualized by spraying the chromatograms with either 0.5 N NaOH in 50% ethanol followed by 1% $AgNO_3$ in acetone or a fresh mixture of 2% sodium metaperiodate and 1% $KMnO_4$ (4:1 v/v) in 2% aqueous Na_2CO_3 (18). Amino acids were visualized with ninhydrin. This chromatographic procedure separates glucose from fructose but not from galactose and mannose (17).

In some cases two-dimensional chromatography was used to examine the eluates from the Dowex 1 borate columns. The first dimension was developed in Solvent I as above, and the second dimension was developed in 10.7 M isobutyric acid:14.8 M ammonium hydroxide:water (66:1:33, v/v/v) (Solvent II) (19). The two-dimensional chromatograms were autoradiographed with Kodak SB5 x-ray film for 2–5 months to visualize the location of ^{14}C.

Samples of the eluates from the Dowex 1 formate columns, which

[2] The time for completion of the phosphorylation reaction was determined in parallel incubation mixtures to which $NADP^+$ (0.05 mM) and yeast glucose-6-P dehydrogenase (0.9 units/ml) were added and the time course of the reaction monitored by the fluorometric assay of NADPH formation.

were obtained after derivatization of the glucose in the Dowex 1 borate column eluates to glucose-6-P with hexokinase or to gluconic acid with glucose oxidase (see below), were examined for radiochemical purity by ascending TLC on cellulose plates developed with Solvent II. These chromatograms were also dried, cut into strips, and assayed for ^3H and ^{14}C contents as above.

Amino Acid Analyses—Amino acid contents of chromatographic column eluates and other fractions derived at various stages in the purification procedures were measured with a Beckman 121-A amino acid analyzer.

Modified Procedure B for Isolation of Glucose—The metabolism of [2-^3H,U-^{14}C]glucose beyond fructose-6-P produces a variety of products that have lost the ^3H but not the ^{14}C labels. We therefore added steps to remove labeled cationic or neutral metabolites from the Dowex 1 borate column eluates. After removal of the borate as methyl borate the Dowex 1 borate column eluates were acidified to pH 2.9–3.0 and applied to Dowex 50-H^+ columns (0.8 × 10 cm). The columns were washed with 18 ml of water and then eluted with 15 ml of 1 N HCl. Both the effluent and eluted fractions were dried separately under a stream of nitrogen at 65–70 °C, redissolved in water, and assayed for ^3H and ^{14}C contents as above.

Glucose oxidase was selected to derivatize the glucose because its specificity for glucose is greater than that of hexokinase (11, 12, 20, 21). To remove neutral contaminants from the fraction containing the glucose, 0.8-ml portions of the effluents from the Dowex 50-H^+ columns were incubated with glucose oxidase (20 units/ml) and catalase (1000 units/ml) in 0.12 M sodium phosphate, pH 7.0, for 90 min at 37 °C (22). Approximately 98% of the glucose was converted to gluconic acid, as determined by disappearance of glucose measured by fluorometric assay of hexokinase and glucose-6-P dehydrogenase-dependent NADPH formation (14). The reaction mixtures were then cooled to 4 °C and applied to Dowex 1 formate columns (0.8 × 9 cm), which were then washed with 27 ml of water and eluted with 10 ml of 8 N formic acid. The effluents and the eluates were dried under a stream of nitrogen at 65–70 °C, dissolved in water, and assayed for ^3H and ^{14}C content as above. Portions of each of the eluates from the Dowex 1 formate columns, which contained the gluconic acid derived from the glucose, were chromatographed on cellulose TLC plates in parallel with authentic gluconic acid standards and samples of the [2-^3H,U-^{14}C]glucose injection solution that were mixed with neutralized extracts of brain and carried in parallel through the isolation procedure. The chromatograms were developed in Solvent II, air-dried, cut into strips, and assayed for ^3H and ^{14}C contents as above.

Alternate Procedure for Isolation of Radiochemically Pure Glucose from Brain—A second, more rigorous procedure to isolate and purify glucose from brain was applied to a few rat brains to confirm the values for the $^3H/^{14}C$ ratios obtained in the gluconic acid derivatives separated by the modified Procedure B. Neutralized acid extracts were prepared separately from duplicate samples of brain of one rat killed at 3 min and another at 5 min, and from single brain samples of two rats killed at 9 min after the pulse of [2-^3H,U-^{14}C]glucose. Glucose was isolated in parallel from portions of each of these samples by both the modified Procedure B and by the following alternate procedure. Portions (0.5 ml) of neutralized acid extracts of brain labeled *in vivo* (and also similar extracts of unlabeled brain containing added samples of the injection solution) were applied to Dowex 1 formate columns (0.8 × 9 cm) which were then washed with 15 ml of water. The combined effluent and wash fractions were adjusted to a pH of 2.9–3.0 and then applied to Dowex 50-H^+ columns (0.8 × 9 cm). The columns were washed with 4 ml of water, and the combined effluent and wash fractions were dried under a stream of nitrogen at 55 °C, redissolved in water, and applied to silica gel TLC plates impregnated with 0.2 M sodium acetate. The chromatograms were developed with isopropanol:ethyl acetate:water (7:1:2, v/v/v) (23); this system separates glucose from galactose and mannose but not from fructose. Glucose standards were chromatographed in parallel. The chromatograms were divided into sequential 0.5–1.0-cm segments, and the silica gel was scraped off each segment and eluted with water. The glucose-containing fractions were identified by fluorometric assay of hexokinase and glucose-6-P dehydrogenase-dependent NADPH formation in portions of the eluates from each segment (14). To minimize the possibility of contamination from compounds hidden in the skirts of the glucose peak, only the fraction positioned at the center of the distribution of glucose in the various segments was rechromatographed on silica gel exactly as above. The silica gel was scraped off consecutive segments of the chromatogram and eluted with water, and the position of the glucose peak was identified as above. A portion of the fraction containing the glucose was assayed for ^3H and ^{14}C contents as above. Another portion was purified further by derivatization to gluconic acid with glucose oxidase by the same procedure described above, except that the reaction was run at a lower temperature (21 °C) for a shorter time (30 min) to minimize oxidation of possible contaminants that might serve as substrates for glucose oxidase; under these conditions about 90% of the glucose was converted to gluconic acid. The reaction mixture was then applied to a Dowex 1 formate column (0.8 × 10 cm), which was then washed with 25 ml of water and eluted with 10 ml of 8 N formic acid. The eluate was dried under nitrogen at 50–55 °C, dissolved in water, and assayed for ^3H and ^{14}C content as above.

RESULTS

Time Course of $^3H/^{14}C$ Ratio in Eluates from Dowex 1 Borate Columns—As reported by Huang and Veech (1), there was a time-dependent fall in the $^3H/^{14}C$ ratio (relative to that in the injectant) in the Dowex 1 borate eluates derived from brain by their Procedure A. This fall exceeded that in the eluates from plasma (Fig. 1). As they also reported (43), the addition of the derivatization step (*i.e.* the hexokinase-catalyzed derivatization of glucose) in Procedure B did not remove the time-dependent reduction in the $^3H/^{14}C$ ratio; if anything, the decline appeared to be even greater (Fig. 1).

^{14}C-Labeled Contaminants in Eluates of Dowex 1 Borate Columns—Nelson *et al.* (13) used a different, more extensive and rigorous procedure to isolate and derivatize glucose from brain and could not confirm the time-dependent fall in the $^3H/^{14}C$ ratios in brain exceeding that in plasma. In an attempt to resolve this discrepancy, we carefully examined the radiochemical purities of the glucose and glucose-6-P fractions obtained by Procedures A and B. The Dowex 1 borate eluate was the final step in the purification of glucose obtained by the original procedure of Huang and Veech (1) (Procedure A), and the Dowex 1 formate eluate was the final step in the purification of glucose-6-P obtained by their modified procedure (43) (Procedure B). Autoradiography of two-dimensional chromatograms of Dowex 1 borate eluates prepared from

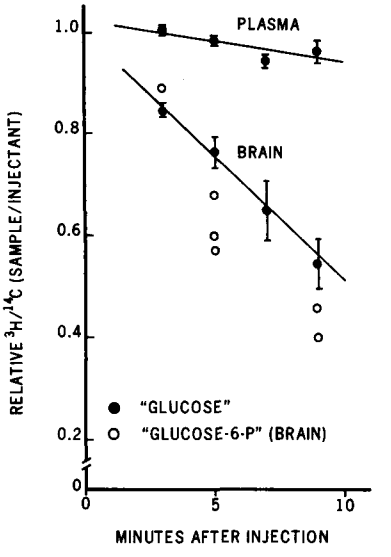

FIG. 1. **Time courses of $^3H/^{14}C$ ratios of glucose and glucose-6-P fractions isolated according to Procedures A and B, respectively.** The $^3H/^{14}C$ ratios are expressed relative to that obtained when a sample of the [2-^3H,U-^{14}C]glucose injectant solution was mixed with a neutralized acid extract of nonradioactive brain and carried through the isolation procedures in parallel with the experimental brain and plasma samples. Values for glucose are the mean ± S. D. of 5, 6, 4, and 3 rats at 3, 5, 7, and 9 min, respectively; the values for glucose-6-P are from individual rats drawn from the above groups.

brains of rats killed, for example, 5 min after the [^{3}H,^{14}C] glucose pulse demonstrated at least seven ^{14}C-labeled compounds contaminating the [^{14}C]glucose (Fig. 2*A*, *top panel*). The quantitative distributions of the ^{3}H and ^{14}C labels among these compounds were determined from scintillation counting of sequential segments of one-dimensional chromatograms of the Dowex 1 borate eluates. Typical chromatograms from brains 5 and 9 min and from arterial plasma 9 min after the [^{3}H,^{14}C]glucose pulse are presented in the *lower panels* of Fig. 2*A*. ^{3}H and ^{14}C were not distributed proportionately among the various components in the chromatograms from brain. The component with the same R_F as the glucose standard contained less than 40% of the total ^{14}C recovered from the entire chromatogram but had a ^{3}H/^{14}C ratio of 9.4, the same as that of the injectant and of the glucose recovered from chromatograms of the injectant (Fig. 2*B*, *lower panel*) or of extracts of plasma (Fig. 2*A*, *lowest panel*).

A variable but always considerable fraction of the ^{14}C recovered from the one-dimensional chromatograms of all the Dowex 1 borate eluates, whether from brain extracts, plasma, or injection solution, was recovered in a contaminant with a mobility like that of methylglucoside. Its ^{3}H/^{14}C ratio, however, was the same as that of the components migrating like glucose (Fig. 2, *A* and *C*). This contaminant was not found in the chromatograms of the injection solution that had not been subjected to the Dowex 1 formate/Dowex 1 borate column chromatography (Fig. 2*B*, *lower panel*) and was presumed to be methylglucoside formed from the glucose when the dried Dowex 1 borate eluates were redissolved in methanol and evaporated to dryness at 70–75 °C to remove the volatile methyl borate (see "Experimental Procedures"). Methylglucoside is readily formed from methanol and glucose under acidic conditions with heating (24, 25) and would be expected to have the same ^{3}H/^{14}C ratio as the glucose from which it was artifactually derived during the isolation procedure.

There were at least six additional ^{14}C-labeled, detritiated contaminants in the Dowex 1 borate eluates from brain extracts (Fig. 2*A*, *upper panel*) that migrated on TLC quite differently from methylglucoside and glucose. Their ^{3}H/^{14}C ratios were markedly lower than those of the methylglucoside

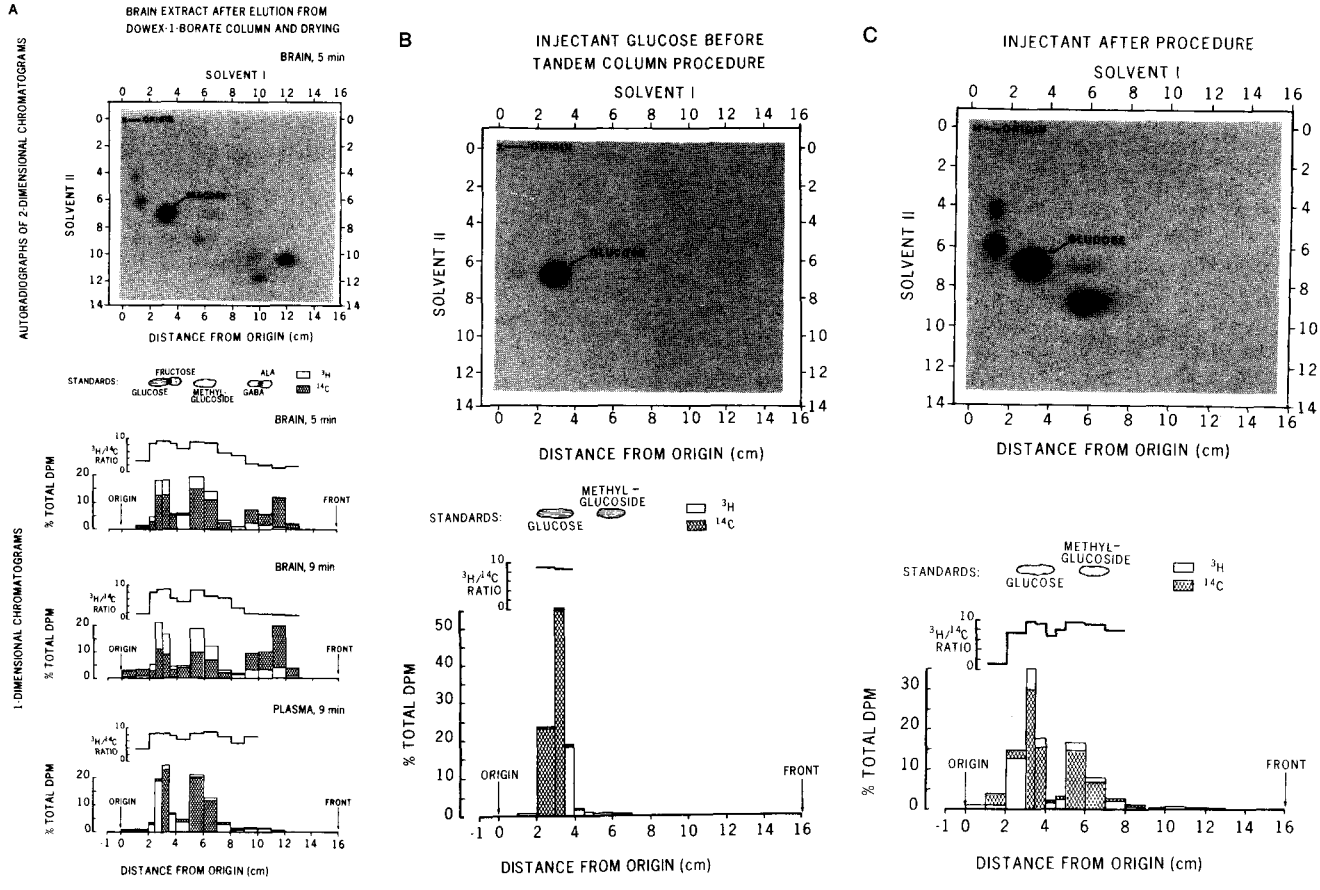

FIG. 2. **Chromatographic analysis of the radiochemical purity of the Dowex 1 borate column eluates.** Samples of the Dowex 1 borate column eluates were chromatographed by two-dimensional TLC on cellulose with Solvents I and II for the first and second dimensions, respectively (*top panels*), and by one-dimensional TLC with Solvent I (*bottom panels*). Labeled compounds were visualized by autoradiography of the two-dimensional chromatograms. The one-dimensional chromatograms were cut into 1 × 0.5 or 1.0-cm strips which were counted in a liquid scintillation counter. The ^{3}H/^{14}C ratios and ^{3}H and ^{14}C contents in the spots in the two-dimensional chromatograms are similar to those with the same R_F in Solvent I in both the two-dimensional and one-dimensional chromatograms. *A*, Dowex 1 borate eluates from brain and plasma of rats killed at 5 or 9 min after the pulse of [2-^{3}H,U-^{14}C]glucose. *B*, [2-^{3}H,U-^{14}C]glucose injectant mixed with a neutralized acid extract of nonradioactive brain before the tandem Dowex 1 formate/Dowex 1 borate column chromatographic isolation procedure for glucose; note the trace amount of a single labeled impurity in the original injection mixture. *C*, the injectant solution was added to a nonradioactive brain extract as in *B* but carried through the tandem Dowex 1 formate/Dowex 1 borate column chromatography by Procedure A.

and glucose components or of the injection solution, and they increase in amount and/or proportion with time after the pulse of [2-^{3}H,U-^{14}C]glucose (Fig. 2*A*, *middle panels*). In addition to the methylglucoside three derivatives of glucose were produced by the isolation procedure itself; they were found in the Dowex 1 borate chromatograms when the [^{3}H,^{14}C]glucose injectant was added to neutralized acid extracts of unlabeled brain and then isolated by Procedure A (1) (Fig. 2*C*) but were absent in chromatograms of the untreated injection solution (Fig. 2*B*). These contaminants contained about 20% of the total ^{14}C recovered in the chromatograms; they were sufficient to reduce significantly ($p < 0.001$, t test) the ^{3}H/^{14}C ratio in the Dowex 1 borate eluates isolated from the mixture of the injection solution and unlabeled brain extract from an initial value of 9.40 ± 0.02 to 8.69 ± 0.15 (mean ± S.E., $n = 10$) (Fig. 2*C*).

The thin layer chromatograms of the eluates derived from plasma (Fig. 2*A*, *lowest panel*) showed glucose and methylglucoside constituents with ^{3}H/^{14}C ratios like that of the injectant (Fig. 2*B*, *lower panel*) and little radioactive contamination like that seen in the extracts from brain (Fig. 2, *A* and *C*). The fall in the ^{3}H/^{14}C ratio in the Dowex 1 borate fraction isolated from brain did not, therefore, result from uptake by the brain of detritiated products of [2-^{3}H,U-^{14}C]glucose from the plasma. Instead, it appeared to be the result of the accumulation of detritiated ^{14}C-labeled contaminants, some produced in the brain *in vivo* and some by the isolation procedure.

^{14}C-Labeled Contaminants of Glucose-6-P and Gluconic Acid Derivatives of Glucose—In Procedure B the glucose in the Dowex 1 borate eluates was phosphorylated with hexokinase, and acidic components in the reaction mixture were retained on Dowex 1 formate and then eluted with formic acid (43). This procedure would purify glucose-6-P of labeled contaminants if there were no other labeled acidic components (presumably removed earlier by the initial passage of the neutralized brain extract through the Dowex 1 formate column) nor any other labeled substrates for hexokinase in the Dowex 1 borate eluate. Thin layer chromatography of the Dowex 1 formate eluates revealed a major, largely detritiated but ^{14}C-labeled contaminant with an R_F greater than that of glucose-6-P. The ^{3}H/^{14}C ratio of the glucose-6-P in the chromatograms was similar to that of the injectant (Fig. 3*A*). The derivatization procedure clearly did not remove all labeled impurities from the glucose-6-P fraction.

The possibility was considered that the Dowex 1 borate eluate contained some neutral and/or cationic, detritiated, ^{14}C-labeled metabolites of glucose that were also substrates for hexokinase; if so, they would have co-purified with glucose and its glucose-6-P derivative. The Dowex 1 borate eluates were, therefore, chromatographed on Dowex 50-H$^+$ columns to remove any cationic metabolites (*e.g.* glucosamine) derivatized to gluconic acid with glucose oxidase, a more substrate-specific enzyme for glucose than hexokinase (11, 12, 20, 21). The reaction mixtures containing the gluconic acid derivative were applied to Dowex 1 formate columns, which were eluted with formic acid. A rapidly moving acidic contaminant with a low ^{3}H/^{14}C ratio was still present in thin layer chromatograms of fractions derived from brain; it increased in amount with time after pulse labeling of the rats (Fig. 3*B*). This contaminant was absent in chromatograms of fractions derived from mixtures of injectant and unlabeled brain extracts and from plasma (Fig. 3*B*). The ^{3}H/^{14}C ratio of the gluconic acid component was essentially the same in chromatograms of fractions derived from experimental brains, injectant, or plasma.

Incomplete Recovery of [^{14}C]Glucose-6-P from Dowex 1 Formate Columns—Glucose-6-Pase activity in brain was previously assessed not only from the fall in the ^{3}H/^{14}C ratio in the glucose fractions isolated from brain but also from differences in rates of [2-^{3}H]glucose and [U-^{14}C]glucose utilization (1, 43). These rates were calculated by dividing the ^{3}H and ^{14}C recovered in the ^{3}H-labeled and ^{14}C-labeled metabolite fractions by the specific activities of their respective labeled precursor pools (1, 26). [^{3}H]Water and neutral and cationic metabolites were recovered in the effluents of the tandem Dowex 1 formate/Dowex 1 borate columns. The labeled acidic products (mainly ^{14}C-labeled metabolites) were eluted from the initial Dowex 1 formate columns with 10 ml of 4 N formic acid (1, 26); the same column chromatographic procedure was used to recover the glucose-6-P derivatized from the glucose in the Dowex 1 borate eluates (43). When we assessed recovery of glucose-6-P by this procedure, we found that 10 ml of 4 N formic acid eluted less than 50% of glucose-6-P from Dowex 1 formate columns; higher anion concentrations (*e.g.* 2 N HCl + 2 N NaCl) were needed to achieve complete recovery (Table I).

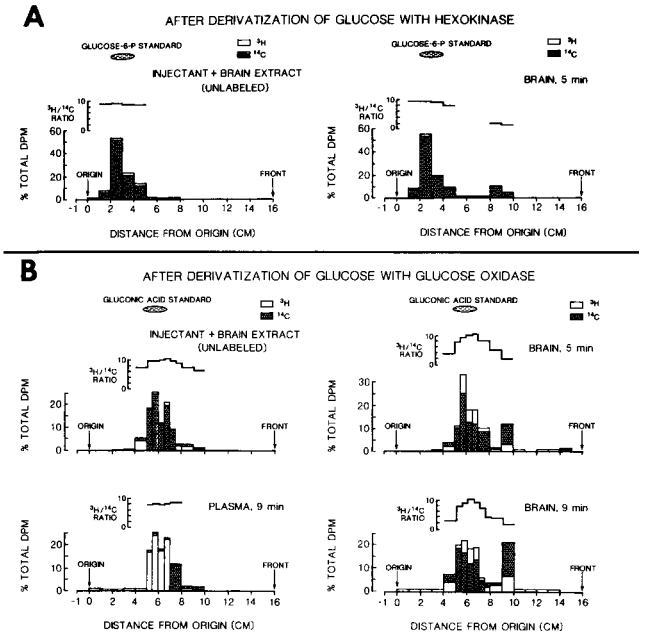

FIG. 3. **Chromatographic analysis of radiochemical purity of glucose-6-P and gluconic acid fractions after derivatization of the glucose in the Dowex 1 borate column eluates by hexokinase or glucose oxidase, respectively.** Labeled acidic contaminants in the crude glucose-6-P and gluconic acid fractions were separated by cellulose TLC in Solvent II, eluted from strips of the chromatograms, and assayed by liquid scintillation counting. *A*, thin layer chromatograms of the crude glucose-6-P fractions obtained by Procedure B. *B*, thin layer chromatograms of the crude gluconic acid fractions obtained by our modification of Procedure B.

Recovery of 2-[^{14}C]deoxyglucose-6-P from Dowex 1 formate columns was a factor in two studies (27, 28) in which [^{14}C]deoxyglucose-6-P formed in rat brain *in vivo* was assayed in fractions isolated from brain extracts by elution with formic acid from Dowex 1 formate columns as above. Kinetic analyses dependent on these data suggested that [^{14}C]deoxyglucose-6-P is rapidly dephosphorylated by glucose-6-Pase activity in brain *in vivo*. We found, however, that 10 ml of 4 N formic acid eluted only about 50% of [^{14}C]deoxyglucose-6-P from the columns (Table I).

Effects of More Extensive Purification of Dowex 1 Borate Column Eluates—Because the derivatization with hexokinase proved inadequate, further steps were added to Procedure B

TABLE I

Incomplete elution of [^{14}C]glucose-6-P and [^{14}C]deoxyglucose-6-P from Dowex 1 formate columns by formic acid

A, [U-^{14}C]glucose was first purified by passage through Dowex 1 formate and Dowex 50-H^+ columns and then derivatized to [^{14}C]glucose-6-P by incubation for 50 min at room temperature in the following reaction mixture: 0.1 mM carrier glucose; 0.5 μCi of [U-^{14}C]glucose; 0.28 units of hexokinase; 80 mM Tris, pH 8.1; 0.8 mM $MgCl_2$; and 0.25 mM ATP in a final volume of 1 ml. Portions of the reaction mixtures (0.4 ml) were applied to Dowex 1 formate columns, washed with 15 ml of water to remove [^{14}C]glucose, and [^{14}C]glucose-6-P was eluted by sequential additions of 10 ml of 4 N formic acid and 23 ml of 2 N HCl + 2 N NaCl. The two eluates were collected separately, and portions of each eluate and the effluent/wash fraction were assayed for ^{14}C contents by liquid scintillation counting. Results are shown for a representative experiment; in 6 experiments, the recovery (mean ± S.D.) of [^{14}C] glucose-6-P eluted by 4 N formic acid was 39.4 ± 2.7%, and an additional 60.5 ± 3.4% was recovered by subsequent elution with 2 N HCl + 2 N NaCl. B, rats were injected intravenously with 50 μCi of [^{14}C]deoxyglucose and killed by freeze-blowing 45 min later. Neutralized acid extracts of brain were prepared (see "Experimental Procedures"). Portions (0.5 or 0.25 ml) of the extracts were applied to Dowex 1 formate columns (5 × 0.8 or 2.5 × 0.8 cm, respectively), washed with 15 ml of water to remove [^{14}C]deoxyglucose, and [^{14}C]deoxyglucose-6-P was eluted first with 10 ml of 4 N formic acid and then 23 ml of 2 N HCl + 2 N NaCl and assayed by liquid scintillation counting. In a second replicate experiment, the recoveries of ^{14}C in the effluent fractions and the 4 N formic acid and 2 N HCl + 2 N NaCl eluates were 13.4, 42.1, and 43.3%, respectively, for the 5-cm column and 13.3, 66.6, and 21.6%, respectively, for the 2.5-cm column.

Fraction	A. [^{14}C]Glucose-6-P		B. [^{14}C]Deoxyglucose-6-P			
	5 × 0.8-cm column[a]		5 × 0.8-cm column[a]		2.5 × 0.8-cm column[a]	
	dpm	*%*	*dpm*	*%*	*dpm*	*%*
dpm applied to column	448,273	100	29,915	100	14,978	100
Effluent (hexose)	6,375	1.4	4,003	13.4	2,012	13.4
Eluate (hexose-6-P)						
1. 10 ml 4 N formic acid[a]	187,225	41.8	12,544	41.9	10,298	68.8
2. 23 ml 2 N HCl + 2 M NaCl	250,925	56.0	13,552	45.3	2,312	15.4
Recovery		99.2%		100.6%		97.6%

[a] Previous studies used 4 N formic acid to elute ^{14}C-metabolites of [2-^{3}H,U-^{14}C]glucose (1, 26) or [^{14}C]deoxyglucose (27, 28) formed in brain; because the dimensions of the Dowex 1 formate columns were only specified in one report (1), two sizes were tested in the present study.

to purify the glucose in the Dowex 1 borate eluates. First, cationic ^{14}C-labeled contaminants (comprising approximately 5% of the total ^{14}C in fractions from brain) were removed by Dowex 50-H^+ column chromatography. Second, glucose in the effluents from the Dowex 50-H^+ columns was derivatized with glucose oxidase to gluconic acid, and the reaction mixtures were applied to Dowex 1 formate columns. The effluents contained neutral impurities, such as other sugars known to bind to Dowex 1 borate (8, 9), glycogen (10), and methylglucoside (Fig. 2), that are not converted to acidic products by glucose oxidase. These neutral contaminants had low $^3H/^{14}C$ ratios and accounted for approximately 11% of the total ^{14}C applied to the Dowex 1 formate columns. Finally, the gluconic acid was eluted from the Dowex 1 formate columns and separated by TLC (Fig. 3*B*). The $^3H/^{14}C$ ratios of the gluconic acid isolated by this procedure from brain were not statistically significantly lower than those in the gluconic acid isolated similarly from plasma (Figs. 3*B* and 4*C*); the p values (paired t test) were >0.05 at 3, 7, and 9 min after the pulse, and at 5 min the ratios in brain were actually significantly higher than those of plasma ($p < 0.05$). The $^3H/^{14}C$ ratios in the gluconic acid derived from both brain and plasma did show small progressive declines with time following the pulse of [2-^{3}H,U-^{14}C]glucose, but the slopes of their regression lines were not significantly different from each other ($p > 0.05$; see the legend to Fig. 4*C*). Thus, each step added to the procedure to remove labeled impurities from the Dowex 1 borate column eluates led to a corresponding increase in the $^3H/^{14}C$ ratio until the ratios in the fractions purified from brain equaled those from the plasma (Fig. 4*C*).

Characterization of Contaminants in the Dowex 1 Borate Column Eluates—Most of the detritiated, ^{14}C-labeled contaminants in the Dowex 1 borate eluates from brain co-purified on Dowex 1 formate columns with the glucose-6-P and gluconic acid derivatives of the glucose in the eluates (Figs. 2*A* and 3), indicating that they were acidic compounds. The major acidic contaminant in the chromatograms of the Dowex 1 formate eluates after the derivatization procedure had the same R_F as that of the major contaminant in the two-dimensional chromatograms of the Dowex 1 borate eluates before derivatization (Fig. 2*A*, *top panel*, Solvent II). This acidic contaminant was found only in extracts from brain and not in those from plasma or injectant (Fig. 4*B*). It had a low $^3H/^{14}C$ ratio (20% of that of the injectant) that remained relatively constant with time following the pulse of [^{3}H,^{14}C] glucose (Fig. 4*A*). The contribution of this contaminant to the total ^{14}C content in the Dowex 1 borate eluates derived from brain increased strikingly with time after the pulse, partly because of its accumulation (Fig. 4*B*) and partly because of the diminishing contribution of the [^{3}H,^{14}C]glucose as it was metabolized with time. This contaminant was the major cause of the time-dependent decline in the $^3H/^{14}C$ ratio in the impure glucose or derivatized glucose-6-P and gluconic acid fractions, and its removal, for example, from the crude gluconic acid fraction (sequence step 3, Fig. 4*C*) by TLC led to significant increases in the $^3H/^{14}C$ ratios of the final purified gluconic acid fractions ($p < 0.05$, paired t tests between sequence steps 3 and 4 at 3, 5, 7, and 9 min, Fig. 4*C*).

Source of the Contaminants in the Dowex 1 Borate Column Eluates—The fractions of the neutralized brain extracts applied to the Dowex 1 borate columns in Procedures A and B were effluents from the preceding tandem Dowex 1 formate columns and should have had all acidic components removed. It was unlikely, therefore, that the major acidic contaminant was formed in the brain. It was more likely that it was derived in the purification procedure from a neutral or basic metabolite of glucose that had passed through the Dowex 1 formate column, was retained on the Dowex 1 borate column, and

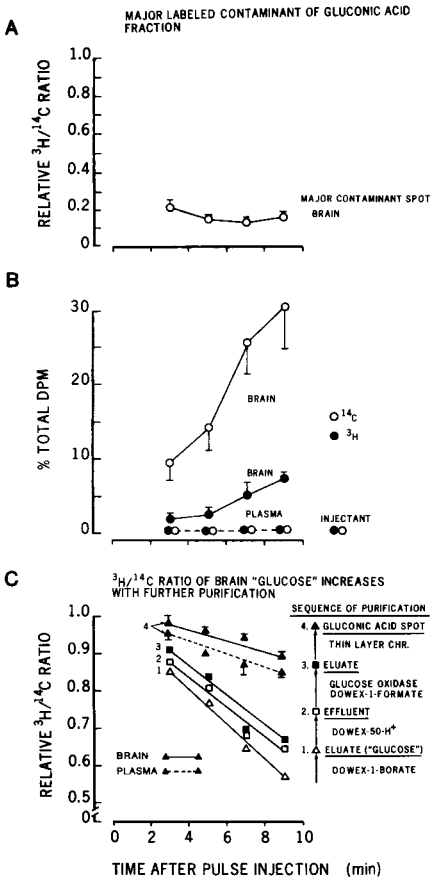

FIG. 4. **Effects of removal of ^{14}C-labeled impurities from Dowex 1 borate column eluates on the $^3H/^{14}C$ ratios of the glucose fractions isolated from brain at each step in our modified Procedure B.** Values are the mean ± S.E. for 5, 6, 4, and 3 rats at 3, 5, 7, and 9 min, respectively (S.E. not shown if smaller than symbol). *A*, time course of $^3H/^{14}C$ ratio of the most prominent acidic contaminant in the gluconic acid fraction derived from brain (see Fig. 3*B*, *right panels*). Note lack of change with time; the slope of the regression line was not statistically significantly different from zero ($p > 0.24$). *B*, time-dependent incorporation of ^3H and ^{14}C into the most prominent acidic contaminant of the gluconic acid fractions isolated from brain and plasma (see Fig. 3*B*, *right panels*). The slopes of the linear regression equations were statistically significantly greater than zero in the fractions from brain ($p < 0.001$ for both ^3H and ^{14}C), showing increasing contributions from this contaminant to the total ^3H and ^{14}C contents in this fraction with time; there was no significant change in the fraction derived from plasma ($p > 0.41$). *C*, time courses of $^3H/^{14}C$ ratios in brain fractions at each step in the purification by our modified Procedure B. The $^3H/^{14}C$ ratios are expressed relative to those obtained in fractions at the same stage of the purification procedure from the mixture of [2-^{3}H,U-^{14}C]glucose injectant and neutralized acid extract of nonradioactive brain. The $^3H/^{14}C$ ratio of the injectant was 9.4 in the initial brain extract prior to the isolation procedure, 8.7 ± 0.2 ($n = 10$) in the Dowex 1 borate eluate, 8.9 ± 0.1 in the Dowex 50-H^+ effluent fraction, 9.2 ± 0.1 in the Dowex 1 formate eluate fraction after glucose oxidase-catalyzed derivatization of glucose to gluconic acid, and 9.5 ± 0.1 in the gluconic acid spot after cellulose TLC in solvent II ($n = 5$ for all injectant fractions after the Dowex 1 borate step). For clarity in the illustration, S.E. values are omitted for purification steps 1–3; the coefficients of variation for these purification stages ranged from 3 to 19% for the Dowex 1 borate eluates (sequence step 1), 3 to 15% for the Dowex 50-H^+ effluents (sequence step 2), and 2 to 3% for the Dowex 1 formate eluates (sequence step 3). There were statistically significant increases in the $^3H/^{14}C$ ratios of the gluconic acid spots (sequence step 4) derived from brains of rats killed at all times after the pulse-labeling when the acidic contaminants were removed from the crude gluconic acid fractions (sequence step 3) by TLC ($p < 0.05$, paired t test). The ratios of the final gluconic acid fractions (sequence step 4) derived from brain were not significantly different from those derived from plasma at 3, 7, and 9 min, but the ratio in the fraction from brain was actually statistically significantly higher than that from plasma at 5 min ($p < 0.05$, paired t test). The slopes of the linear regression lines of the $^3H/^{14}C$ ratios of the gluconic acid spots for both plasma and brain (sequence step 4) were both negative and statistically different from zero. The regression line for brain was $Y = 1.02 - 0.013\,X$ (S.E. of slope ±0.003, $p = 0.002$); the regression line for plasma was $Y = 1.01 - 0.019\,X$ (S.E. of slope ±0.004, $p = 0.004$). The slopes of the regression lines for brain and plasma were not statistically significantly different from each other ($p > 0.5$).

then eluted from this column along with the glucose. Neutral amino acids are known to pass through Dowex 1 formate columns at neutral pH (29–31). With the exception of serine and threonine, which are known to form borate complexes (32), other neutral amino acids might not be expected to be retained on Dowex 1 borate columns. Amino acid analyses of the Dowex 1 borate eluates derived from brain demonstrated, however, that virtually all the neutral amino acids were present (Fig. 5*A*). To check further whether the purification procedure removed neutral amino acids, tracer amounts of representative ^{14}C-labeled neutral amino acids and glucosamine were added individually to neutralized acid extracts of unlabeled rat brain, and 0.1 or 0.5 ml of the mixture was passed through the tandem Dowex 1 formate/Dowex 1 borate columns. With the 0.5-ml samples, 93 ± 2% of glutamine, 91 ± 1% of alanine, 84 ± 2% of leucine, and 62 ± 8% of glucosamine (mean ± S.E., $n = 3$) were retained by the Dowex 1 borate column and released by elution with formic acid. With the 0.1-ml samples, 88 ± 4% of glutamine was retained ($n = 3$).

The amino acids present in the Dowex 1 borate eluates may have also contributed to some of the less prominent acidic, detritiated, ^{14}C-labeled contaminants in the eluates (Figs. 2, 3, and 5*B*). Carbonylamino addition compounds of amino acids and reducing sugars were probably formed during the glucose isolation procedure.[3] Amino acids are known to react with glucose and other aldoses to form *N*-substituted glycosylamines. These can spontaneously undergo Amadori rearrangements to 1-amino-1-deoxy-2-ketoses, reactions which would cause loss of ^3H from [2-^{3}H,U-^{14}C]glucose. The detritiated, ^{14}C-labeled products can, especially with heating, undergo complex dehydration and fission decomposition reactions, *e.g.* the Maillard or "browning" reactions which are known to produce more than 15 compounds from glucose and ammonia or glycine alone (33, 34). No attempt was made to identify the products or assess the magnitude of these reactions in the present study. Some of the detritiated, ^{14}C-labeled contaminants seen in the chromatograms of the Dowex 1 borate eluates derived from processing mixtures of the injectant with neutralized acid extracts of unlabeled brain through Procedure A (Fig. 2*C*), were probably products of these types of reactions[3]; their contribution may be reflected in the statistically significant decrease ($p < 0.001$, t test) in the $^3H/^{14}C$

[3] Tracer amounts of [U-^{14}C]glucose and a ^3H-labeled representative neutral amino acid, [^{3}H]leucine, were added to a neutralized, acid extract of nonradioactive brain and subjected to the same purification procedure through the Dowex 1 borate column chromatography, derivatization with hexokinase, and separation of the acid products of the derivatization procedure on Dowex 1 formate just as was applied to the experimental brain extracts. TLC of the eluates from the Dowex 1 formate columns revealed easily identified clearly separated spots for [^{14}C]glucose and [^{3}H]leucine, but in addition ^3H and ^{14}C were found to co-migrate to several spots 5–9 cm from the origin with R_F values corresponding to those of the minor components of the acidic, detritiated ^{14}C-labeled contaminants in the chromatograms of the Dowex 1 borate eluates from the experimental brain extracts (Figs. 3 and 5*B*). This co-migration was not found in chromatograms of the [^{14}C]glucose/[^{3}H]leucine/brain extract mixture prior to the isolation procedure.

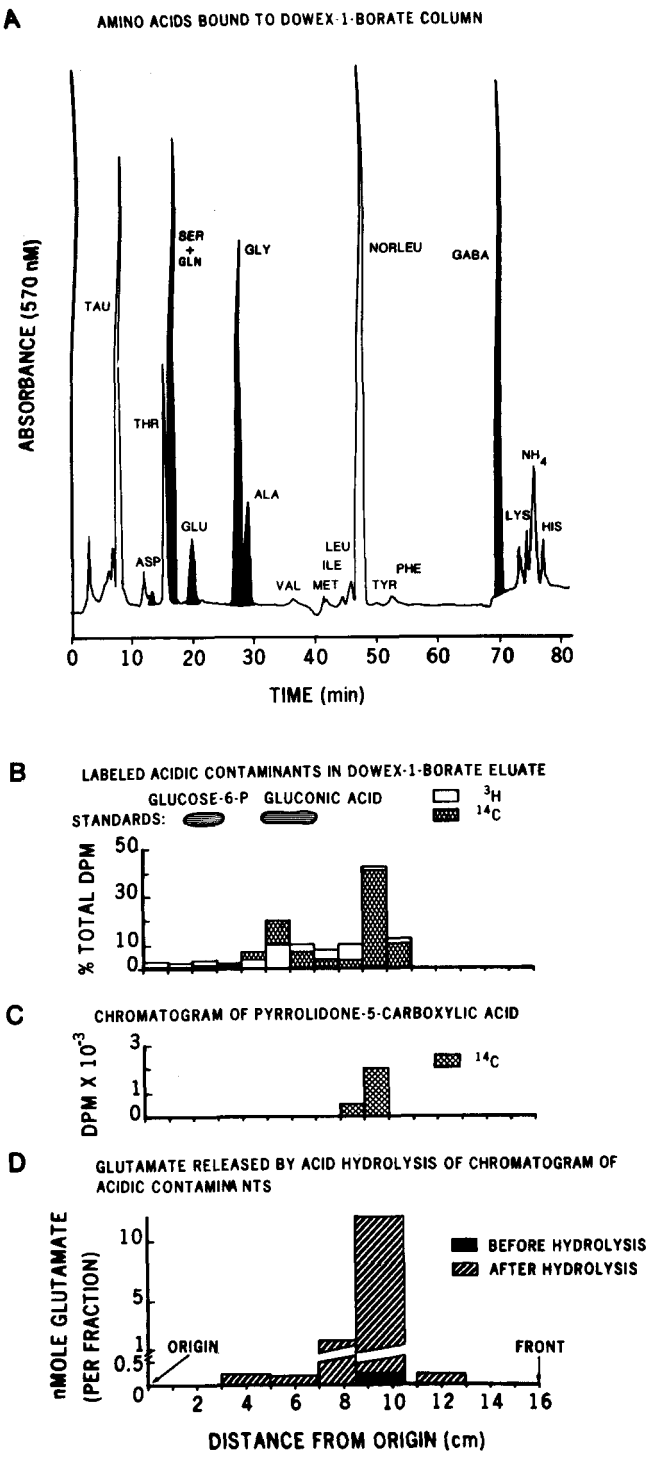

FIG. 5. **Identification of major contaminants of crude glucose, glucose-6-P, and gluconic acid fractions.** *A*, amino acids in Dowex 1 borate column eluates. The chromatogram is representative of five, four from individual rats and one from the pooled eluates from three rats. The retention of amino acids by the Dowex 1 borate column was essentially proportional to the amount of neutralized extract applied to the tandem column over the range of 0.1 to 1.0 ml (based on 14 analyses of the same pooled Dowex 1 borate eluate). Norleucine was added as an internal standard. Amino acids derived from glucose metabolism (*i.e.* those that would be ^{14}C-labeled after the pulse of [2-^{3}H,U-^{14}C]glucose) are *shaded*. Serine and glutamine were not separated and are reported as the sum of their concentrations; because the glutamine was virtually entirely cyclized to pyrrolidone-5-carboxylic acid at the Dowex 1 borate step (see "Results"), this sum represents mainly the serine concentration. *B*, labeled acidic impurities in Dowex 1 borate eluate. Dowex 1 borate eluates were pooled from all rats killed 5 min after the pulse of [2-^{3}H,U-^{14}C] glucose, and acidic compounds in these eluates were separated by sequential Dowex 50-H$^+$ and Dowex 1 formate column chromatography and TLC according to our modified Procedure B, except that the glucose oxidase step was omitted. The ^{3}H/^{14}C ratios (normalized to that of the injectant) of all these acidic compounds eluted from each strip were very low, *e.g.* < 0.3. The most prominent contaminant located 10–11 cm from the origin has an R_F similar to that of the major contaminants in the two-dimensional chromatogram of the Dowex 1 borate column eluate (Fig. 2*A*, *top panel*, second dimension, Solvent II) and those in the crude glucose-6-P (Fig. 3*A*) and crude gluconic acid (Fig. 3*B*) fractions. Glucose-6-P and gluconic acid were not present in the sample chromatographed in *B*, but standards of each are included to facilitate comparison of the ^{14}C-labeled acidic contaminants present in the Dowex 1 borate eluate fraction chromatographed here with those in the crude glucose-6-P (Fig. 3*A*) and gluconic acid (Fig. 3*B*) fractions. The detritiated ^{14}C-labeled acidic contaminants with the same R_F as gluconic acid would have copurified with the gluconic acid derivative of glucose in the chromatogram in Fig. 3*B*, but since the ^{3}H/^{14}C ratio of the gluconic acid spot on the thin layer chromatogram was not depressed below that of the plasma (Fig. 4*C*) their contribution must have been negligible. *C*, R_F of pyrrolidone-5-carboxylic acid. [^{14}C]Pyrrolidone-5-carboxylic acid was synthesized by heating an aqueous solution of 1 mM L-[^{14}C] glutamine for 1 h at 100 °C (37, 38). After cooling, the sample was adjusted to pH 3 and applied to a Dowex 50-H$^+$ column to remove any glutamine and glutamate. The Dowex 50-H$^+$ column effluent was chromatographed on cellulose in Solvent II. Identical results were obtained when authentic [^{14}C]glutamine was added to a neutralized acid extract of nonradioactive brain and processed according to our modified Procedure B. *D*, acid hydrolysis of pyrrolidone-5-carboxylic acid to glutamate. Acidic impurities in the Dowex 1 borate column eluate were isolated and chromatographed as in *B* above. The chromatograms were cut into 1 × 2-cm strips, eluted with water, and centrifuged to remove the cellulose. Prior to amino acid analysis, one-half of each sample was heated in 2 N HCl for 2 h at 100 °C to hydrolyze pyrrolidone-5-carboxylic acid to glutamate (37, 38). The results are representative of two such experiments.

ratio of the injectant from its initial value of 9.40 ± 0.02 to 8.69 ± 0.15 (n = 10) in the Dowex 1 borate eluate.

Identity and Origin of Major Acidic Contaminant—The major detritiated, ^{14}C-labeled contaminant in the Dowex 1 borate eluates (Fig. 3, *A* and *B*) could not be one of the neutral amino acids because it was not removed by Dowex 50-H$^+$, and acidic compounds originally present in the brain extracts should have been removed on the initial Dowex 1 formate columns. The major contaminant must, therefore, have been formed during the purification procedure. Its R_F on the chromatograms was the same as that of a 2-pyrrolidone-5-carboxylic acid standard (Fig. 5, *B* and *C*).

Detritiated [^{14}C]glutamine is a major product of [2-^{3}H,U-^{14}C]glucose metabolism in brain (29). When heated in aqueous solution under neutral or acidic conditions, as in the drying of the Dowex 1 borate eluates (1), glutamine rapidly cyclizes to pyrrolidone-5-carboxylic acid with release of the amide nitrogen as ammonia; small amounts of glutamate can also be formed (29, 35–38). When tracer amounts of authentic [^{14}C]glutamine were added to unlabeled brain extracts which were then processed by Procedure A (1) to the Dowex 1 borate eluate stage, 93 ± 2% (n = 3) of the ^{14}C was recovered in the eluate, and 98 ± 1% (n = 2) of this recovered ^{14}C could not be retained on Dowex 50-H$^+$, suggesting nearly complete conversion of glutamine to an acidic or neutral compound. When the effluents from the Dowex 50-H$^+$ columns were carried through the glucose oxidase procedure used to derivatize glucose and chromatographed on Dowex 1 formate columns, TLC of the eluates from these columns showed that essentially all the ^{14}C migrated with the same R_F as authentic pyrrolidone-5-carboxylic acid (Fig. 5, *B* and *C*). The principal acidic contaminant found in chromatograms of the glucose-6-P and gluconic acid derivatives (Procedure B) also had the same R_F (Fig. 3, *A* and *B*). Acid hydrolysis (*e.g.* heating in 2

N HCl at 100 °C for 2 h) is known to convert pyrrolidone-5-carboxylic acid to glutamate quantitatively (37, 38). Dried Dowex 1 borate eluates obtained from unlabeled brain extracts to which [^{14}C]glutamine had been added contained very little ^{14}C that was retained by Dowex 50-H$^+$ before and 98% following acid hydrolysis. When thin layer chromatograms of the acid components in the Dowex 1 borate eluates (separated from the Dowex 1 borate eluates by sequential Dowex 50-H$^+$ and Dowex 1 formate column chromatography) were cut into successive 1 × 2-cm segments, eluted with water, subjected to acid hydrolysis, and assayed for glutamate content by amino acid analysis, the principal acidic component in the chromatograms was converted to glutamate (Fig. 5*D*). Finally, amino acid analyses of the fractions taken at various further stages of purification of dried Dowex 1 borate eluates derived from brain extracts demonstrated large increases in glutamate concentration in all of the samples after acid hydrolysis (Table II). These results (Fig. 5, Table II) indicate that ^{14}C from [2-^{3}H,U-^{14}C]glucose was incorporated into glutamine, which then cyclized during the drying of the Dowex 1 borate eluate to pyrrolidone-5-carboxylic acid and became the major acidic, detritiated, ^{14}C-labeled contaminant before or after derivatization of the glucose to glucose-6-P or gluconic acid. Release of small amounts of other amino acids by acid hydrolysis (Table II) is consistent with the formation of *N*-addition compounds.[3]

Confirmation of Absence of Selective Detritiation of [2-^{3}H, U-^{14}C]Glucose in Brain by Alternate Purification Procedure—Many of the deficiencies of Procedures A and B were clearly associated with the use of Dowex 1 borate columns. An alternate procedure to purify the glucose from brain, one that completely avoided the use of Dowex 1 borate, was, therefore, employed to check the results obtained with the modified Procedure B (see "Experimental Procedures"). Briefly, anionic and cationic metabolites of [2-^{3}H,U-^{14}C]glucose were first removed from the neutralized acid extracts of brain by sequential Dowex 1 formate and Dowex 50-H$^+$ column chromatography. The effluents were evaporated to remove ^3H_2O. The glucose in the effluents was isolated by two cycles of TLC on silica gel and elution with water and then derivatized to gluconic acid with glucose oxidase. The gluconic acid derivative was isolated by Dowex 1 formate column chromatography

TABLE II

Effect of acid hydrolysis on glutamate concentrations in brain glucose fractions

Amino acid concentrations were measured at various stages of purification of glucose (see "Experimental Procedures"). Prior to amino acid analysis one-half of each sample was hydrolyzed by heating at 100 °C in 2 N HCl for 2 h (37, 38). Serine and glutamine were not separated by the amino acid analytical procedure and are tabulated as the sum of their contents. All of the glutamine present in the Dowex 1 borate column eluate cyclized to 2-pyrrolidone-5-carboxylic acid during the drying step; pyrrolidone 5-carboxylate is converted to glutamate by acid hydrolysis (see Fig. 5*A* and text).

Amino acid	Dowex 1 borate column eluate[a]		Dowex 1 formate column eluate			
	Acid hydrolysis		After Dowex 50-H$^+$ column[b]		After hexokinase[c]	
	Before	After	Acid hydrolysis		Acid hydrolysis	
			Before	After	Before	After
	nmol/fraction					
Serine plus glutamine	3.4	3.5	<0.1	0.5	0.4	1.2
			<0.1	1.3		
			0.3	1.9		
Glutamate	0.4	18.0	<0.1	14.7	0.4	17.1
			<0.1	30.2		
			0.4	24.2		
Glycine	2.4	2.7	<0.1	0.4	0.3	2.3
			<0.1	1.2		
			0.6	4.1		
Alanine	1.5	1.6	<0.1	0.2	<0.1	0.9
			<0.1	0.5		
			<0.1	2.4		

[a] Portions of neutralized acid extracts of nonradioactive brains were pooled and processed according to Procedure A, and the Dowex 1 borate eluates were subjected to acid hydrolysis and assayed for amino acid contents. The concentrations of amino acids before acid hydrolysis are representative of those obtained in six such experiments (Fig. 5*A*). The large increase in glutamate concentration after hydrolysis is consistent with the results of the experiment in which authentic [^{14}C]glutamine was added to a neutralized acid extract of a nonradioactive brain and processed according to Procedure A; the ^{14}C-labeled derivative of [^{14}C]glutamine could not be removed from the redissolved Dowex 1 borate eluate by Dowex 50-H$^+$ column chromatography unless subjected first to acid hydrolysis (see text).

[b] Portions of redissolved Dowex 1 borate eluates from 2, 6, or 8 rats were combined into 3 pools. The acidic contaminants in each of these pools were separated as follows. Each sample was acidified to pH 3, applied to a Dowex 50-H$^+$ column (0.8 × 9 cm) and washed with 25 ml of water. The combined effluent and wash fractions, now free of amino acids, were dried at 50 °C in a stream of nitrogen, redissolved in water, adjusted to pH 7.0–7.5, and applied to a Dowex 1 formate column (0.8 × 5 cm), washed with 15 ml of water, and eluted with 10 ml of 8 N formic acid. The eluates were dried as above, redissolved in water, subjected to acid hydrolysis, and assayed for amino acid contents.

[c] Portions of the Dowex 1 borate eluates from two rats were pooled and incubated with hexokinase as described in Procedure B. The incubation mixture was applied to a Dowex-1 formate column (0.8 × 10 cm), washed with 18 ml of water, and eluted with 15 ml of 23 N formic acid. The eluate was dried at 75 °C in a stream of air, redissolved in water, subjected to acid hydrolysis, and assayed for amino acid contents. Results presented are from one experiment; in two other experiments, similarly small amounts of amino acids were detected before acid hydrolysis.

and assayed for ^3H and ^{14}C by liquid scintillation counting. The $^3H/^{14}C$ ratios in the gluconic acid recovered from brains of rats killed 3, 5, and 9 min after the pulse were constant in the last two purification steps (indicating that constant specific activity had been attained) and were essentially the same as those found in the final gluconic acid fractions purified from other portions of the same brains by the modified Procedure B (Table III). These results confirm by an entirely independent method that there is no greater rate of loss of ^3H than of ^{14}C from [2-^{3}H,U-^{14}C]glucose in brain, at least none greater than accounted for by changes in the $^3H/^{14}C$ ratio in the plasma glucose supplied to the brain.

DISCUSSION

The present study demonstrates that the observations interpreted by Huang and Veech (1, 43) as evidence of rapid dephosphorylation of glucose-6-P by glucose-6-Pase in rat brain *in vivo* were artifacts arising from deficiencies in the procedures used to purify glucose and/or its derivatized products from brain. Significant glucose-6-Pase activity, if present, would indeed cause a time-dependent fall in the $^3H/^{14}C$ ratio in the glucose pool in the tissue following its pulse labeling with [2-^{3}H,U-^{14}C]glucose; it would also lead to a higher rate of utilization of [2-^{3}H]glucose than of [U-^{14}C]glucose. It was findings like these that led to the conclusion that glucose-6-P is dephosphorylated at a rate as high as one-third that of its rate of formation in rat brain *in vivo* (1, 43). We were able to reproduce these findings with the purification procedures used in those studies but found that the fall in the $^3H/^{14}C$ ratio in the glucose fraction isolated from brain was due not to a change in the ratio in the true glucose pool in the brain but to contamination of this fraction with detritiated, ^{14}C-labeled products derived from the glucose, either from its metabolism or the purification procedure. When glucose was purified from brain by more rigorous procedures, its $^3H/^{14}C$ ratio did not fall below that seen in the plasma glucose being supplied to the brain. There was, therefore, no need to invoke glucose-6-Pase activity.

Most of the problems with those purification procedures (1, 43) arose from excessive reliance on sequential Dowex 1 formate/Dowex 1 borate column chromatography to bind glucose selectively and separate it from the numerous detritiated, ^{14}C-labeled metabolic products of [2-^{3}H,U-^{14}C]glucose metabolism (*e.g.* all metabolites from fructose-6-P onward). The present studies demonstrate that many of the steps in those procedures do not behave exactly as expected. 1) The initial Dowex 1 formate column does not remove all detritiated ^{14}C-labeled metabolites of [2-^{3}H,U-^{14}C]glucose that can bind to Dowex 1 borate. 2) Dowex 1 borate does not selectively bind only glucose but also retains many of the neutral or cationic detritiated ^{14}C-metabolites, such as neutral amino acids or other sugars. 3) The elution of labeled acidic metabolites, particularly [^{14}C]hexosephosphates, from the Dowex 1 formate columns with 4 N formic acid is incomplete. 4) The procedure used to remove formic and boric acids from the Dowex 1 borate eluates produces acidic derivatives of detritiated, ^{14}C-labeled products of [2-^{3}H,U-^{14}C]glucose metabo-

TABLE III

$^3H/^{14}C$ ratios in glucose fractions isolated by different procedures

At each experimental time, glucose was purified in parallel from portions of neutralized acid extracts of brains of rats killed at the indicated times by Procedure A, Procedure B, our modified Procedure B, and by the alternate procedure that avoided the use of the Dowex 1 borate column (see "Experimental Procedures"). The $^3H/^{14}C$ ratios are expressed relative to that of the injectant of [2-^{3}H,U-^{14}C]glucose mixed with a nonradioactive brain extract and carried in parallel through the same isolation procedures.

Purification step	Relative $^3H/^{14}C$ ratios at various times					
	3 min[a]		5 min[a]		9 min[b]	
	Sample 1	Sample 2	Sample 1	Sample 2	Rat 1	Rat 2
A. Tandem Dowex 1 formate/Dowex 1 borate column chromatography						
Dowex 1 borate column eluate[c]	0.66 ± 0.06	1.00 ± 0.01	0.83 ± 0.04	0.76 ± 0.01	0.58 ± 0.04	0.52 ± 0.04
B. Further purification of glucose[d]						
Crude glucose-6-P fraction (hexokinase derivatization)	0.41	0.82	0.36	0.47	0.32	0.28
Crude gluconic acid fraction (glucose oxidase derivatization)	0.85	0.98	0.91	0.80	0.76	0.57
Gluconic acid spot on TLC plate	0.98	0.94	0.95	0.95	0.93	0.88
Alternate purification procedure[e]						
Glucose spot on TLC plate	0.98	0.97	0.95	0.99	0.94	0.87
Gluconic acid derivative of glucose	0.98	1.02	0.97	0.98	0.90[f]	

[a] Values of duplicate samples from one brain.

[b] Values are those obtained from two different rats.

[c] The values are the mean ± S.D. of samples processed in triplicate or quadruplicate from each of the duplicate samples of one brain and from each rat.

[d] The 3–4 replicate Dowex 1 borate eluates for each sample were recombined, and portions were purified further in parallel by Procedure B (derivatization of glucose to glucose-6-P with hexokinase and recovery of the crude glucose-6-P fraction as the eluate of a Dowex 1 formate column) and by our modified Procedure B (Dowex 50-H^+ column chromatography, derivatization of glucose to gluconic acid with glucose oxidase, recovery of the crude gluconic acid fraction as the eluate of a Dowex 1 formate column, and separation of the gluconic acid by TLC on cellulose with Solvent II).

[e] The two neutralized acid extracts described in Footnotes *a* and *b* were processed by the alternate purification procedure (see "Experimental Procedures"), *i.e.* sequential Dowex 1 formate and Dowex 50-H^+ column chromatography, drying to remove ^3H_2O, TLC on silica gel with isopropanol:ethyl acetate:water (7:1:2; v/v/v) as the solvent, elution of the glucose spot, rechromatography on silica gel, and elution of the glucose spot again. The $^3H/^{14}C$ ratio was determined in a portion of the glucose, and the remainder was derivatized to gluconic acid with glucose oxidase and isolated by Dowex 1 formate column chromatography.

[f] $^3H/^{14}C$ ratio in pooled samples from both rats combined after elution from the TLC plate.

lism that subsequently co-purify with the glucose-6-P separated on Dowex 1 formate columns after derivatization of the glucose in the Dowex 1 borate eluates with hexokinase. 5) The same procedure converts some of the [^{3}H,^{14}C]glucose to methylglucoside which cannot be phosphorylated by hexokinase and thus artifactually reduces the proportion of derivatized [^{3}H,^{14}C]glucose to that of acidic, ^{14}C-labeled, detritiated contaminants in the derivatized fraction; this artifact is further enhanced by the less complete recovery of the [^{14}C]glucose-6-P derivative than of the contaminants with 4 N formic acid elution of the Dowex 1 formate columns.

A major source of the problems with the Dowex 1 borate step of the purification procedure (1) is the passage of neutral amino acids in the brain extracts through the initial Dowex 1 formate column. These include not only unlabeled neutral amino acids normally present in brain, but also detritiated, ^{14}C-labeled amino acid products of [2-^{3}H,U-^{14}C]glucose metabolism, mainly glutamine, γ-aminobutyric acid, and alanine. These amino acids bind to the Dowex 1 borate along with the [^{3}H,^{14}C]glucose. Reactions between the [2-^{3}H,U-^{14}C]glucose and unlabeled amino acids, *e.g.* browning reactions (32, 33), may occur during the warming used to evaporate the formic and boric acids from the Dowex 1 borate eluates and contribute to the detritiated ^{14}C-labeled contaminants produced by the purification procedure itself (Fig. 2, Table III).[3] The warming also cyclizes the detritiated [^{14}C]glutamine in the eluates to [^{14}C]pyrrolidone-5-carboxylic acid, thus regenerating an acidic contaminant in this fraction after all acidic metabolites had been removed by the previous Dowex 1 formate column chromatography. It is mainly this acidic contaminant, which co-chromatographs on Dowex 1 formate with the glucose-6-P derivatized from the glucose in the Dowex 1 borate eluates, that lowers the ^{3}H/^{14}C ratio (Fig. 5); its effect on the ratio is enhanced because not all the [^{3}H,^{14}C]glucose (*i.e.* the portion converted to methylglucoside) is phosphorylated, and only about one-half of the glucose-6-P is eluted from the Dowex 1 formate column (Table I).

The presence of labeled glutamine and γ-aminobutyric acid in the Dowex 1 borate eluates was recently acknowledged (26), and a Dowex 50-H$^+$ column step to remove them was added to the previous procedures (1, 43). The decline in the ^{3}H/^{14}C ratio and the difference in rates of [2-^{3}H]glucose and [U-^{14}C]glucose utilization were diminished but still present. It was not specified where in the procedure the cation exchange column was inserted (26). If it were after the Dowex 1 borate column step, it would not have removed the acidic contaminants demonstrated in the present study, *e.g.* pyrrolidone-5-carboxylic acid and the other acidic compounds.

The same deficiencies in the purification procedure that cause the fall in the ^{3}H/^{14}C ratio are also responsible for the apparent higher rates of [2-^{3}H]glucose than of [U-^{14}C]glucose utilization in brain, particularly at early times (1, 26). The rate of utilization for each radioactive species of glucose is calculated by dividing the total amount of radioactive metabolites formed from that labeled species by the specific activity of its precursor pool. The total amount of product formed in the brain during the entire experimental period must be recovered for the calculation to be valid. These calculations are, therefore, valid only for very short periods, *e.g.* within a minute or less, after the pulse because ^3H_2O and $^{14}CO_2$ are rapidly formed by [2-^{3}H]glucose and [U-^{14}C]glucose metabolism, respectively, and lost from the brain. It is obvious that the defects in the purification procedures that were used (1, 26, 43) would lead to erroneously low calculated rates of [U-^{14}C]glucose utilization at all times. First, all of the ^{14}C-metabolites are not recovered in the product pool because some (*e.g.* ^{14}C-labeled neutral amino acids, etc.) are recovered instead in the fraction representing the precursor pool, and others (*i.e.* sugar phosphates) are not quantitatively eluted and recovered from the Dowex 1 formate columns. Second, the estimated specific activity of the precursor [^{14}C]glucose pool is too high because of the ^{14}C-labeled contaminants (some derived from metabolites and others produced by the purification procedure) in the Dowex 1 borate eluates in which it is assayed. With too low a numerator and too high a denominator, the calculated rate of [^{14}C]glucose utilization must be too low. This would be true at all times, even at zero time when the effects of the detritiated, ^{14}C-labeled derivatives of [2-^{3}H,U-^{14}C]glucose produced by the browning reactions would still be present.

On the other hand, quantitative recovery of the ^{3}H-labeled metabolites of [2-^{3}H]glucose in the brain is relatively simple because its main labeled metabolite, ^3H_2O formed at the glucose-6-P isomerase step, is easily recovered in the effluents from the tandem Dowex 1 formate/Dowex 1 borate columns. The other [^{3}H]metabolites are eluted from the Dowex 1 formate and Dowex 1 borate columns and comprise only a small fraction of the total; inaccuracies in their recoveries would have relatively little effect on total product recovery. Also, the specific activity of the [^{3}H]glucose determined in the Dowex 1 borate eluates is much closer to the true specific activity of the [^{3}H]glucose precursor pool than is the case with the [^{14}C]glucose because of the relatively low level of ^{3}H-labeled contaminants. The rate of glucose utilization calculated with the [^{3}H]glucose is, therefore, much closer to the true rate and higher than the rate calculated with [^{14}C]glucose. This is true only at very early times after the pulse of [2-^{3}H,U-^{14}C]glucose (less than a minute) because the early loss of the ^3H_2O product from brain rapidly reduces the rate of glucose utilization calculated with [^{3}H]glucose down toward the already erroneously low levels calculated with [^{14}C]glucose (1, 26). Like the change in the ^{3}H/^{14}C ratio, the difference in the rates of [2-^{3}H]glucose and [U-^{14}C]glucose utilization (1, 26) was not due to glucose-6-Pase activity but rather to deficiencies in the purification procedures.

High rates of dephosphorylation of the 2-[^{14}C]deoxyglucose-6-P formed in rat brain during the first 45 min (27) or between 12 and 24 h (28) after an intravenous pulse of 2-[^{14}C]deoxyglucose were concluded on the basis of nonlinear least squares best fits to measured time courses of the [^{14}C]deoxyglucose and [^{14}C]deoxyglucose-6-P concentrations in the brains of groups of rats. These results were interpreted as evidence of significant glucose-6-Pase activity in brain (27, 28). The validity of the fits depend on accurate values for the [^{14}C]deoxyglucose and [^{14}C]deoxyglucose-6-P concentrations. Aside from other problems addressed previously (40), in both studies the [^{14}C]deoxyglucose-6-P concentrations were determined in eluates from Dowex 1 formate columns eluted with 4 N formic acid, a procedure found in the present studies to fail to elute and recover hexosephosphates quantitatively (Table I). It is likely, therefore, that the loss of [^{14}C]deoxyglucose-6-P that was attributed in those studies (27, 28) to glucose-6-Pase activity in rat brain was, at least in part, due to loss on the Dowex 1 formate columns instead.

The claims (1, 26, 27, 43) of high levels of glucose-6-Pase activity in brain *in vivo* were clearly inconsistent with the low levels repeatedly found when this enzyme was assayed *in vitro* (2–5). The present studies demonstrate that deficiencies in the purification procedures fully account for those findings without any need to implicate glucose-6-Pase activity. Speculation about the existence and functional significance of futile cycles in cerebral energy metabolism stimulated by these claims (41, 42) was premature. There is no need at present to revise our current concepts of glucose metabolism in brain.

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